Ca-ATPase (SERCA) through adjusting the membrane surface charge. We used EPR to measure the backbone dynamics of TOAC spin-labeled PLB, time-resolved (TR) FRET to measure the interprobe distance between the donor IAEDANS in SERCA and the acceptor Dabcyl in PLB, and NADH-linked ATPase assay to measure PLB's inhibitory function. The cytoplasmic domain of PLB has a net charge of +3 under physiological conditions. When free or bound to SERCA in lipid vesicles, PLB is in equilibrium between an ordered T state and a dynamically disordered R state, as measured by EPR. TR-FRET resolved two interprobe distances between SERCA and PLB. Compared to zwitterionic lipids (DOPC), anionic lipids (DOPS or DOPG) increased the population of the T state, which has the longer interprobe distance between SERCA and PLB, and the inhibition of SERCA's ATPase function. Cationic lipids (DOEPC or DOTAP) decreased the T state population and SERCA inhibition. We conclude that electrostatics can tune the structural and functional dynamics of PLB. The ordered T state is oriented closer to the membrane surface, resulting in a longer SERCA-PLB interprobe distance, and is more inhibitory. The disordered R state is oriented away from the membrane surface, resulting in a shorter SERCA-PLB interprobe distance, and is less inhibitory. Modulating the membrane surface charges provides a new way of investigating the correlation between internal structural dynamics and function of membrane proteins.

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Phospholamban does not Interact with *E*2 Intermediates of the Cardiac Calcium Pump Stabilized by Metal Fluorides

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The phospholamban (PLB) monomer binds in the groove between M2, M4, and M9 of the cardiac calcium pump (SERCA2a) and inhibits enzyme activity. We previously demonstrated that cross-linking of PLB with engineered Cys-residues to this region is prevented by micromolar Ca, but is stimulated by the nucleotides ATP or ADP. We proposed that PLB inhibits the catalytic activity of the enzyme by stabilizing a unique, nucleotide bound, Ca-free intermediate of SERCA2a (*E*2●ATP). Stabilization of *E*2●ATP prevents Ca binding at the high affinity Ca binding sites and formation of E1, thus blocking the kinetic cycle. To further test our model, we have now checked if PLB interacts with other E2 conformations, notably, SERCA2a with the bound metal fluorides (MgF_4^{2-}, AlF_4^-, or BeF_3^-), which mimic the *E*2●Pi, *E*2P, and *E*2-P states, respectively. Interactions between PLB and SERCA2a were examined by cross-linking of N30C-PLB to K328 of WT-SERCA2a at the cytoplasmic extension of M4 and of V49C-PLB to V89C-SERCA2a at the C-terminal end of M2. Insect cell microsomes co-expressing N30C-PLB/SERCA2a or V49C-PLB/V89C-SERCA2a were first incubated with the metal fluorides in Ca-free buffer, and then subjected to chemical cross-linking. All these metal fluorides prevented cross-linking of PLB to SERCA2a at both the cytoplasmic and transmembrane regions, suggesting that PLB does not interact with *E*2●Pi, *E*2P, and *E*2-P formed in the absence of nucleotide. Remarkably, however, the cross-linking of PLB to SERCA2a was completely restored upon addition of 3mM ATP, allowing formation of *E*2●ATP. These results strongly support our model in which *E*2●ATP is the single conformation binding PLB, and further suggest that the binding interaction between *E*2●ATP and PLB is very strong, sufficient to prevent formation of *E*2●Pi, *E*2P, or *E*2-P, even when stabilized by metal fluorides.

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Interaction of SERCA with the Transmembrane Domain of Phospholamban Measured by FRET in Live Cells

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Phospholamban (PLB) is the key regulator of the SERCA calcium pump. Previous quantitative fluorescence resonance energy transfer (FRET) measurements between Cerulean-SERCA and YFP-PLB have shown that calcium and thapsigargin reduce the affinity of the PLB-SERCA binding interaction. Though a structure change in the regulatory complex would account for this reduced affinity, we detected no change in FRET distance. We hypothesized that in the presence of calcium the transmembrane domain of PLB moves to a secondary site on SERCA without altering the positions of the PLB cytosolic domain or fluorescent protein fusion tag. To test this hypothesis, we engineered a truncated PLB construct (tmPLB) composed of a fluorescent protein fused to the transmembrane domain of PLB (residues 28-52). We observed FRET from Cerulean-SERCA to YFP-tmPLB, but with a significantly longer FRET distance compared to full-length PLB. SERCA-tmPLB FRET was not significantly changed by saturating concentrations of calcium or thapsigargin. Notably, we did not detect inhibition of SERCA by YFP-tmPLB using an in-cell calcium uptake assay, even though other groups have previously shown that the PLB transmembrane domain can inhibit SERCA activity. In addition, oligomerization of the tmPLB was detected by intrapentameric FRET. Compared to full-length PLB, the oligomerization affinity of tmPLB was normal, but we measured a shorter FRET distance as a result of deletion of the cytoplasmic domain. The results demonstrate that tmPLB retains some normal protein-protein interactions despite its apparently anomalous interaction with SERCA. Taken together, the results suggest a non-inhibitory binding interaction between YFP-tmPLB and SERCA, perhaps as a result of steric hindrance by the YFP fusion tag. It is also possible that the truncated PLB binds selectively to a non-inhibitory site on SERCA. Experiments are underway to address these unresolved questions.

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Evidence of Direct Binding of G-Actin and Calmodulin to PMCA by Surface Plasmon Resonance

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Previous studies of our laboratory suggest that the main isoform of the plasma membrane calcium ATPase (PMCA) from erythrocytes, PMCAh4b, associates with the actin cytoskeleton and its catalytic activity can be regulated through this interaction. Apparently, G-actin and/or short oligomers enhance the catalytic activity of the pump, while F-actin seems to inhibit it. To further explore the interaction between PMCA and G-actin, we performed binding experiments based on Surface Plasmon Resonance technology (SPR) using purified PMCAh4b from erythrocytes and purified rabbit muscle G-actin on a BIAcore T100 system. Our strategy consisted in immobilizing G-actin to the sensor chip surface while PMCA constituted the analyte (125 nM to 2 μM). The sensorgrams obtained showed an increase in the response as a function of PMCA concentration with a saturable binding. The unspecific binding values were subtracted from the response obtained in the control flow cell (without immobilized G-actin). The apparent KD (1.25 μM) was determined by kinetic analysis with parameters kon (1.33 ± 0.07) 104 M-1s-1 and koff (1.66 ± 0.03) 10-2 s-1.

Conversely, we immobilized PMCAh4b to the sensor chip surface. The analytes were (i) G-actin (0.6 to 5 μM) or (ii) Calmodulin, a known activator of PMCAh4b (1.8 to 15 nM). Results show that G-actin binds to the activated sensor with KD=3μM, kon (2.98 ± 0.45) 104 M-1s-1 and koff (8.98 ± 0.67) 10-2 s-1 and calmodulin binds with an apparent KD of (1.14 ± 0.6) 10-8 M. This last value agrees with the known value of calmodulin binding affinity determined by other methods.

These results show a specific and high apparent affinity binding between PMCA and G-actin and reveal the usefulness of SPR technology to determine binding constants between small molecules and a large membrane protein like the plasmatic calcium pump. With grants of ANPCYT/CONICET/UBACYT.

3601-Pos Board B462

Conformational changes in the First Transmembrane Helix of the H^+-ATPase, AHA2, during H^+ Transport

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Conformational changes in the first transmembrane alpha helix of the Arabidopsis H^+ pump, AHA2, were studied with a cysteine scanning mutagenesis technique when the enzyme was expressed in *Saccharomyces*. Cysteine-reactive compounds, including N-ethylmaleimide (NEM), fluorescein maleimide (FM), positively (MTSET) and negatively charged methanethiosulfonate (MTSES) reagents, were applied to intact yeast that were actively respiring (non-starved) or cultured overnight at 4°C in glucose-free media (starved). When yeast were exposed to FM, extracellular accessibility of substituted cysteines was determined by measuring fluorescent protein on PAGE gels. In respiring yeast, FM bound only to residues predicted to be in the extracellular 1-2 loop of AHA2; however, in starved yeast, FM binding was observed at residues predicted to be in the extracellular 1-2 loop and deep within the first transmembrane alpha helix, down to residue 73. Pre-incubation with other cysteine-reactive agents showed that MTSET only bound to residues in the extracellular 1-2 loop and MTSES did not bind at all in this area of the enzyme. These results suggest that the conformation of the first transmembrane